Eradication of *Salmonella* Typhimurium in broiler chicks by combined use of P22 bacteriophage and probiotic

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Abstract

It has been reported that the phage therapy is effective in controlling the number of colony-forming unit (CFU) of *Salmonella* spp. in chicken gut. This paper describes the protective effect of phage and *Lactobacilli* administration on *Salmonella* infection in 1-day-old chicks. We administered the bacteriophage P22 in a single dose and a probiotic mixture of four species of bacteriocin-producing *Lactobacillus* once a day for one week. Samples were analyzed every 48 hours, and intestinal eradication of *S. Typhimurium* was confirmed after treatments. We observed an increase in the size of duodenal villi and cecal crypts, as well as an increase in body weight in groups that received daily doses of *Lactobacilli*. This study confirms the efficiency of bacteriophage therapy in controlling salmonellosis in chicks and the beneficial effect of *Lactobacilli* mixtures in the weight gain of the birds.

Introduction

Bacteriophages are host-specific viruses that infect bacteria and are not able to damage either eukaryotic cells or other than their target-bacteria.¹⁻⁵ P22 is a lambdoid bacteriophage species that infects *Salmonella* serogroups A, B, and D1; such group can be identified by the trisaccharide sequence of the somatic antigen α -D-mannose-(1 $\rightarrow$ 4)- α -L-rhamnose-(1 $\rightarrow$ 3)- α -D-galactose-(1 $\rightarrow$ 4). This phage attaches to the target-cell's wall, subsequently cleaving the glycosidic linkage rhamnose-(1 $\rightarrow$ 3)- α -D-galactose due to tailspike enzymes with endoglycosidic activity.³ P22

uses such enzymes to perforate the cell's wall by injecting its DNA into *Salmonella* in order to replicate.⁴ After virion production, lytic bacteriophages cause the rupture of the host cells.⁵

Salmonella are rod-shaped, Gram-negative bacteria that belong to the family *Enterobacteriaceae*. There are three recognized species of *Salmonella*: *S. subterranea*, isolated from soil, *S. bongori*, typically isolated from cold-blooded animals, and *S. enterica*, isolated from warm-blooded animals. The latter is composed by six subspecies (*enterica*, *salmamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*) and approximately 2500 serovars, including the bird-specific types Pullorum and Gallinarum.⁶⁻⁹ *Salmonella enterica* subspecies *enterica* serovars Enteritidis and Typhimurium are usually present in birds as the main cause of paratyphus in Brazil and USA, the latter serovar also being responsible for food-borne infection in man.¹⁰⁻¹² Other serovars commonly isolated are Agona, Anatum, Cubana, Handar, Heidelberg, Mbandaka, Montevideo, and Senftenberg.¹²

The prevention of *Salmonella* infections can be performed by using probiotics, which reduce the number of these pathogenic bacteria with considerable success. *Lactobacillus* is probably the most important genus of lactic acid bacteria used for probiotic production in poultry industry. These bacteria have several strategies to inhibit or even kill other bacterial species, such as the direct competition for linkage sites on the epithelial surfaces and also for nutrients available at the intestinal lumen.¹³ Additionally, there is a release of several metabolic products with antibacterial activity that increase the efficiency of *Lactobacillus* in competing with other microorganisms, i.e. lactic and acetic acid,^{14,15} which modify the luminal pH consequently difficulting the survival of other bacteria; hydrogen peroxide,¹⁶ which affects proteins and destroys nucleic acids from other bacteria; and the production of the so called bacteriocins, which are complex proteins that result from the catabolism of the lactic acid with bacteriocidal effect.¹⁷

The worldwide increase in bacterial resistance stimulated the search for alternative strategies to control salmonellosis. The use of phage therapy and probiotics for such purpose is highlighted since they offer advantages such as the lack of residual substances remaining in animal products and absence drug resistance.¹⁸ Accordingly, the aim of this study was to verify the efficiency of the combined administration of the P22 bacteriophage and a *Lactobacilli*-based probiotic as an experimental treatment in order to eradicate *Salmonella* in chicks. Additionally, we also aimed to verify possible alterations in intestinal microenvi-

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Key words: bacteriophage, intestinal microbiology, probiotic, *Salmonella*.

Acknowledgments: we thank the São Paulo Research Foundation (Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP) for providing the master grant 07/54012-9 to Guilherme Augusto Marietto Gonçalves and the research grant 08/51034-4 for Raphael Lúcio Andreatti Filho.

Received for publication: 20 March 2011.

Revision received: 21 May 2011.

Accepted for publication: 21 May 2011.

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Microbiology Research 2011; 3:e2
doi:10.4081/mr.2011.e2

ronment morphology and changes in birds' weight gain.

Materials and Methods

Birds

Three hundred eighty-four, one-day-old, *Salmonella* free chicks, lineage Avian Farm (*Gallus gallus domesticus*) were housed at the avian center of the Avian Pathology Laboratory, Department of Veterinary Clinical Sciences, School of Veterinary Medicine and Animal Science and Animal Sciences, São Paulo State University – UNESP, Botucatu, Brazil. The study was approved by the Ethics Committee on Animal Experimentation of the School of Veterinary Medicine and Animal Science, UNESP, Botucatu, Brazil. Antibiotic-free food and water were provided *ad libitum*. Confirmation of the absence of *Salmonella* spp. in the birds was evaluated before use through microbiological tests. Twelve extra birds were euthanized by cervical dislocation and the liver, caecum and yolk sacs were aseptically collected. The organs were then incubated overnight at 37°C in tubes containing 10mL of Tetrathionate Broth (TB; Merck, Darmstadt, Germany) and Selenite-Cistine Broth (SCB; Merck, Darmstadt, Germany). The enriched samples were plated on Brilliant Green Agar

(BGA; Oxoid, Basingstoke, England) and Xylose-Lysine Desoxycholate Agar (XLD; Oxoid, Basingstoke, England) and incubated overnight at 37°C. Each plate was evaluated for the presence or absence of lactose negative colonies.

Culture and preparation of *Salmonella* Typhimurium inoculums

We used a laboratory induced nalidixic acid-resistant strain of ST from the Avian Pathology Laboratory, UNESP, Botucatu, Brazil. The initial culture was produced by adding 3 mL of turbid broth of ST in 50 mL of nutrient broth and incubated at 40°C for 24 hours. Then, ST colony-forming units (CFU) were calculated immediately before inoculation by performing decimal serial dilutions using phosphate-buffered saline (PBS) pH 7.2. The diluted sample (0.1mL) was placed in Petri plates containing BGA supplemented with 100 µg/mL of nalidixic acid, and cultivated for 24 hours at 40°C. Birds in groups St, St+Pb, St+Ph and St+Pb+Ph were intraesophageal gavage with 0.5 mL of culture containing 4×10^4 CFU/mL of ST.

Culture and use of the probiotic

The probiotic was formulated using *Lactobacillus acidophilus*, *L. fermentum*, *L. reuteri*, and *L. salivarius* strains from the Avian Pathology Laboratory, UNESP, Botucatu, Brazil. Strains were initially overnight incubated using DeMan-Rogosa-Sharpe medium (MRS; Oxoid, Basingstoke, England) in a jar with Anaerobac System (Probac do Brasil Ltda., Sao Paulo, Brazil) at 37°C, and then placed in MRS medium and overnight reincubated in anaerobiosis at 37°C. The inhibitory effect of the *Lactobacilli* strains on ST was confirmed using the Spot-on-the-lawn method, as described by Lima *et al.*¹⁹ *Lactobacilli* species were individually cultivated every 24 hours, and mixed immediately before inoculation. Decimal serial dilutions of *Lactobacilli* cultures were performed in order to quantify CFU using the same technique previously described for ST, except for the use of MRS as the medium and 37°C as temperature set. The birds of groups Pb, St+Pb, St+Pb+Ph and Pb+Ph were treated every 24 hours by intraesophageal gavage using 0.5 mL containing 6×10^9 CFU/mL of probiotic.

Culture and use of the bacteriophage

A sample of P22 bacteriophage (ATCC 19585-B1) from the Brazilian Agricultural Research Corporation (EMBRAPA), Embrapa Food Technology research center – Rio de Janeiro, Brazil – was used. For P22 culture, a nutrient broth (Acumedia, Lansing, USA) 1 mL solution with 0.5% of NaCl containing 2×10^{10}

P22 CFU and 3 mL of turbid pre-culture containing ST in tryptone soy broth (TSB) (Merck, Darmstadt, Germany) at double concentration were added to 5 mL of TSB with subsequent homogenization. After four hours at 40°C, the solution was filtered using a 0.22 µm porosity micro-filter (Medical Millex-GS filter unity, Millipore, Billerica, USA). Subsequently, quantification of plaque-forming units (PFU) was performed in order to obtain P22 concentration. For such purpose, 100 µL serial dilutions of the P22 filtrate (i.e. 10^{-1} to 10^{-10}) were placed in polypropylene microtubes containing 0.9 mL of PBS, subsequently discharging 100 µL of solution from the last recipient. Posteriorly, 1mL of a pre-culture of ST in TSB at double concentration was diluted in 9 mL of PBS. The total volume of P22 each dilution and 100 µL of ST 1:9 were placed in tubes with 3 mL of TSB soft agar (warmed in boiling bath at 50°C), homogenized, distributed in sterile Petri plates, and kept at room temperature until solidification, using the technique by Debartolomeis and Cabelli²⁰ modified by the authors. Before PFU quantification, plates were overnight incubated at 40°C. Subsequently, birds of groups Ph, St+Ph, St+Pb+Ph and Pb+Ph were inoculated by intraesophageal gavage with 0.5 mL of the mixture containing 3×10^{10} PFU/mL of P22.

Inoculums

Birds were divided into eight groups of 48 individuals each. All groups were identified using specific characters (Table 1). Monitoring of *Salmonella* and bacteriophage colonization, evaluation of length of intestinal villi, and weight gain rate measurements were performed during the experiment. Prior of inoculations, all birds were submitted to overnight fasting. In the first day, birds in groups St, St+Pb, St+Ph, and St+Pb+Ph were intraesophageally challenged with *Salmonella* Typhimurium (ST). Three hours later, groups Ph, St+Ph, St+Pb+Ph and Pb+Ph were given by intraesophageal gavage with the bacteriophage inoculums, and groups Pb, St+Pb, St+Pb+Ph, and Pb+Ph were intraesophageally treated with a probiotic. Treatment with probi-

otic was repeated every 24 hours until 144 hours from the start of the experiment. Sampling was performed every 48 hours from the beginning of the treatments: I-0 hour, II-48 hours, III-96 hours and IV-144 hours. During each collection 12 birds were taken from each group in order to perform the tests.

Evaluation of body weight and intestinal villi morphometry

Body weight was obtained using a digital scale with a 500 g limit. Birds were euthanized by cervical dislocation (according to the resolution 714, June 2002, Federal Council of Veterinary Medicine, Brazil) for collection of duodenum and cecum. For morphometrical analysis samples from the second third of the duodenum including the pancreas and from the distal portion of the cecum (blind fundus) were collected. Samples were washed with a continuous injection of Bouin's solution for removing intestinal content, Bouin-fixed, and paraffin-embedded. Posteriorly, three semi-seriated 5 µm-thick section were obtained, hematoxylin and eosin stained and observed under light microscopy. Slides were analyzed with the Zeiss Axio Imager A1 microscope using the Axio Vision Software Rel. version 4.6.1 (Carl Zeiss MicroImaging, Heidelberg, Germany). Ten duodenal villi and cecal crypts were evaluated in each section in order to obtain morphometric results.

Microbiological monitoring

The monitoring for identifying ST and P22 was achieved by using polymerase chain reaction (PCR) and microbiological isolation from cecum and feces. Ceci were collected at pre-determined moments (i.e. I-0 hour, II-48 hours, III-96 hours and IV-144 hours) and feces were daily collected from the bottom of the cages. Cecum and feces were evaluated as pooled samples in order to increase the chances of detection and isolation of ST and P22. Samples were mashed and 2 g were suspended in 25 mL of PBS. The material was vortexed and filtered in order to remove larger particles, and 1mL of the filtered solution was used for DNA extraction using Chelex-100™ (Biorad Laboratories,

Table 1. Identification of the experimental groups related to the treatment.

Group	Treatment	Number of birds
Cr	Control*	48
St	<i>Salmonella</i> Typhimurium	48
Pb	Probiotic	48
Ph	Bacteriophage P22	48
St+Pb	<i>Salmonella</i> Typhimurium and probiotic	48
St+Ph	<i>Salmonella</i> Typhimurium and bacteriophage P22	48
St+Pb+Ph	<i>Salmonella</i> Typhimurium, probiotic and bacteriophage P22	48
Pb+Ph	Probiotic and bacteriophage P22	48

*Group without treatment.

Richmond, USA). The filtered samples were vortexed in polypropylene tubes, followed by centrifugation at 3,000 rpm for 10 min at 5°C. Supernatant was removed and the pellet was discharged. Ten µL of chloroform was added to 100 µL of the supernatant, gently hand-stirred for 30 sec and incubated for 10 min at 5°C. After incubation, the material was centrifuged at 1,000 rpm for 15 min at 5°C, and 30 µL of the supernatant was added to 30 µL of Chelex-100™ at 5%, subsequently obtaining a 1:1 concentration. The solution was incubated in a MasterCycler Gradient thermocycler (Eppendorf, Hamburg, Germany) at 99°C for 10 min and the supernatant containing free DNA was collected. Primers for the reaction were synthesized by Invitrogen Corporation (São Paulo, Brazil) and suspended at concentration of 10pmol. For P22, the SieB gene (forward 5'-ATGGTGGCAGGAGTTAATGC-3' and reverse 5'-CAACAATCCCGAAGCAGT-3) was used with the following amplification program: 1 min at 94°C, 30 cycles of amplification (30 sec at 90°C, 30 sec at 58°C, and 60 sec at 72°C), followed by a final polymerization for 8 min at 72°C, as described by Mikasová *et al.*²¹ For ST the invA gene (forward 5'-TTGTTACGGCTATTTTGACCA-3' and reverse 5'-CTGACTGCTACCTTGCTGATG-3') was used with the following amplification program: 5 min at 94°C, 35 cycles of amplification (30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C) followed by a final polymerization for 4 minutes at 72°C, as described by Swamy *et al.*²² Amplifications were carried out using 5 µL of each sample added to 1 µL of each primer, 12.5 µL of the amplification kit Go Taq Green Master Mix (Promega, Madison, USA), and 5.5 µL of ultrapure water. Microbiological isolation was carried out using the material processed *in natura* i.e. caecum and feces mashed and suspended in PBS. For recovery of ST, 1mL of each suspension was overnight incubated at 37°C in tubes containing 9 mL of TB and SCB. Samples were then plated in BGA and XLD added with 100 µg/mL of nalidixic acid, subsequently being overnight incubated at 37°C. The presence or absence of lactose negative colonies was evaluated on each plate. For recovery of P22 the remaining suspension was filtered using a 0.22 µm microfilter syringe. Then, 1 mL of the filtrate was placed into 5 mL of TSB/3 mL of turbid pre-culture of ST, and incubated for four hours at 40°C. After filtration, 900 µL were placed in 3 mL of TSB soft agar along with 100 µL of 1:9 ST turbid pre-culture in order to confirm the formation of viral plaques.

Statistical analysis

The Scott-Knott test was used to compare statistical differences. Statistical analyses were performed using the SISVAR software (Universidade Federal de Lavras, Brazil) version 4.0 as described by Ferreira.²³

Results

Microbiological evaluation

In all collections, ST was detected by both PCR and microbiologic isolation from the ceca of the St group. ST Fecal shedding was not detected by any of the methods here used. P22 was detected neither in the ceca nor in feces (Table 2).

Weight gain rate

The weight gain rates obtained during the four collections are summarized in Table 3. The groups with similar weights during the first collection did not reveal statistically significant differences. With the exception of the St group, the groups showed continuous weight gain ($P<0.05$). Birds treated with probiotic had a final weight mean higher than the others, such as in the St+Pb group; however, Pb and St+Pb+Ph groups did not reveal statistically significant differences between themselves. The Cr, Ph, St+Ph and Pb+Ph groups gained the same weight ($P>0.05$) and were lighter when compared to the St+Pb, Pb and St+Pb+Ph groups. The St group significantly lost weight ($P<0.05$) at the very beginning of the experiment, but gained weight after the

third collection (96 hours). Nevertheless, the final weight of St group was significantly lower than those of the control group ($P<0.05$).

Intestinal morphometry

Results of the measurement of the duodenal villi and cecal crypts are summarized in Table 4. All of the groups had an increase in the mean size of duodenal villi and cecal crypts. The mean size of those structures in birds treated with *Lactobacillus* (Pb, St+Pb, St+Pb+Ph, and Pb+Ph) exceeded that of the other groups ($P<0.05$). The Cr, Ph, and St+Ph groups revealed no statistically significant differences between themselves ($P>0.05$). Additionally, cecal crypts were significantly shorter in the St group ($P<0.05$) when compared to other groups.

Discussion

Although paratyphus is relatively common in young birds, chicks older than 14 days rarely manifest clinical signs.²⁴ With the exception of birds challenged with *Salmonella*, the chicks did not present clinical signs in this study, i.e. apathy, ruffled feathers, diarrhea, and mortali-

Table 2. Detection of *Salmonella* Typhimurium in caecum using PCR and conventional bacterial isolation (CFU) in the different treatments.

Group	Period after challenge (hours) *			
	0	48	96	144
Cr	- / ND	- / ND	- / ND	- / ND
St	+ / 4×10^3	+ / 2×10^2	+ / 1×10^2	+ / 6×10^1
Pb	- / ND	- / ND	- / ND	- / ND
Ph	- / ND	- / ND	- / ND	- / ND
St+Pb	- / ND	- / ND	- / ND	- / ND
St+Ph	- / ND	- / ND	- / ND	- / ND
St+Pb+Ph	- / ND	- / ND	- / ND	- / ND
Pb+Ph	- / ND	- / ND	- / ND	- / ND

*Twelve birds Pool for each treatment. Negative: -. Positive: +. ND, no detected.

Table 3. Mean of body weight of birds challenged against *Salmonella* Typhimurium and treated with probiotic and bacteriophage P22. Results are expressed in grams (g).

Group	Period after challenge (hours) ^a			
	0	48	96	144
Cr	42.9±2.7Dns ^b	48.8±4.8Ca	65.8±5.3Bb	87.8±7.3Ab
St	39.0±3.4Bns ^c	35.4a±3.3Cb	45.0±4.1Bc	60.3±11.9Ac
Pb	42.6±2.6Dns	47.9±5.4Ca	76.6±9.7Ba	104.9±10.9Aa
Ph	42.5±4.5Dns	52.3±8.3Ca	67.0±5.5Bb	87.3±7.5Ab
St+Pb	41.2±2.8Dns	52.3±6.1Ca	80.4±5.3Ba	115.3±10.4Aa
St+Ph	43.3±3.1Dns	51.7±6.8Ca	63.9±6.7Bb	91.4±7.1Ab
St+Pb+Ph	42.4±3.3Dns	51.1±7.6Ca	74.9±5.8Ba	106.7±8.4Aa
Pb+Ph	42.1a±1.1Dns	47.2±5.3Ca	73.7±8.3Ba	94.7±11.7Ab

^aValues are arithmetic mean±standard deviation; ^bSmall letters compare the means of the treatments in each collection (column) and capital letters compare the means of the treatments during collections (lines). Values in a column and lines with different letters (e.g., a,b and c) differ significantly ($P<0.05$). ^cNS, no significant.

ty. Birds of the St group presented discrete diarrhea in the first and second days after challenge and such diarrhea ceased in the consecutive days. Such results support those from Zhang *et al.*,²⁵ who documented that *Salmonella* inoculums containing 10^4 to 10^7 CFU may cause persistent diarrhea lasting from two to eight days in birds. The presence of clinical signs, microbiologic isolation, and PCR detection of ST from the intestines confirmed the efficiency of the inoculum used in this experiment.

In this study, no P22 recovery from ceca or feces was achieved. In Ph and Pb+Ph groups, which were not inoculated with *Salmonella*, the lack of target cells probably led to the natural elimination of P22 few hours after inoculation. We hypothesize that P22 was not detected in St+Ph and St+Pb+Ph groups after 24 hours due to the eradication of *Salmonella* by the treatments. Since the results obtained by Siqueira *et al.*²⁶ support the idea that bacteriophage detection is a good method for detecting *Salmonella*, it is possible that the absence of P22 indicates the absence of *Salmonella* in the present study. Consequently, such results indicate that P22 was able to eradicate ST from the intestines of birds treated with St+Ph since there was lack of microbiological recovery or PCR detection of either ST or P22.

These results are different from those reported in previous studies^{10,27-29} which revealed decreases in *Salmonella* CFU number when using bacteriophages as experimental treatment. Those authors used non-identified bacteriophages that, despite revealing success in reduction of *Salmonella* population, did not eradicate the pathogen. This feature is characterized by the possibility of using lentogenic bacteriophages, which could result in latency of the used bacteriophages by prophages insertion. Consequently, the *Salmonella* population would only be reduced by bacteriophages in lytic phase which can eradicate the pathogen. Thus, the use of bacteriophages is particularly encouraged in cases in which the bacteriophage species is known and present a lytic cycle, similarly to what was performed in the present study. Accordingly, our results indicate that the elimination of *Salmonella* – which is a major goal for the avian industry – can be achieved instead of the simple reduction in pathogen number.

In this study, we also tested the potential role of *Lactobacilli* probiotic as an experimental treatment in birds inoculated with ST. We found that the St+Pb treatment performed in chicks was associated with *Salmonella* intestinal eradication after oral administration of a *Lactobacilli* mix. The inhibitory action of *Lactobacillus* used as a probiotic for prevention against *Salmonella* has been already demonstrated *in vivo* by other authors with success;³⁰⁻³³ however, despite other authors

Table 4. Mean of body weight of birds challenged against *Salmonella* Typhimurium and treated with probiotic and bacteriophage P22. Results are expressed in grams (g).

Organ	Group	Period after challenge (hours) ^a			
		0	48	96	144
Duodenum	Cr	1256.8±320.4Cns ^b	1335.9±371.2Bb	1612.7±271.2Ans	1211.7±429.2Ans
	St	1215.8±280.4Cns ^c	1278.0±309.4Bb	1533.8±435.6Ans	1697.3±358.5Ans
	Pb	1385.8±249.6Bns	1689.3±379.3Aa	1760.0±450.1Ans	1921.0±490.5Ans
	Ph	1315.5±276.1Cns	1293.7±382.1Bb	1590.4±256.2Ans	1722.9±379.8Ans
	St+Pb	1248.5±289.4Bns	1667.9±349.1Aa	1757.7±351.1Ans	1908.5±454.9Ans
	St+Ph	1285.5±200.9Cns	1256.9±258.6Bb	1610.4±250.9Ans	1745.6±290.1Ans
	St+Pb+Ph	1345.5±344.5Bns	1692.9±342.9Aa	1753.5±436.5Ans	1900.6±374.8Ans
	Pb+Ph	1366.7±256.8Bns	1785.2±301.9Aa	1796.7±277.4Ans	1033.2±310.9Ans
Cecum	Cr	406.4±50.8Cns	523.3±54.3Bb	660.4±58.6Ab	674.3±58.3Ab
	St	419.9±53.4 Bns	429.4±59.8Bc	523.4±51.0Ac	582.5±56.3Ac
	Pb	435.7±57.7Cns	657.9±50.4Ba	793.9± 50.2Aa	806.5±55.6 Aa
	Ph	418.8±50.1Cns	532.10±59.1Bb	624.6±57.4Ab	674.3±58.4Ab
	St+Pb	405.4±52.6Cns	616.2±60.0Ba	693.9±60.5Aa	748.4±57.8Aa
	St+Ph	405.1±63.5Cns	523.1±47.0Bb	614.9±51.7Ab	676.8±51.8Ab
	St+Pb+P	401.1±37.2Cns	616.6±41.4Ba	729.1±57.8Aa	832.9±59.1Aa
	Pb+Ph	412.2±56.7Cns	630.3±50.8Ba	741.4±54.3Aa	784.3± 51.1Aa

^aValues are arithmetic mean±standard deviation. ^bSmall letters compare the means of the treatments in each collection (column) and capital letters compare the means of the treatments during collections (lines). Values in a column and lines with different letters (e.g., a,b and c) differ significantly ($P<0.05$). ^cN, no significant

have documented diminution in *Salmonella* population in birds treated with competitive-exclusion products,³⁴ the use of *Lactobacilli* as treatment – instead of prevention – has not yet been documented.

In the present study, P22 and *Lactobacilli* probiotic mix was able to eradicate ST in the St+Pb+Ph group. Since groups inoculated with ST and treated with P22 (St+Ph) and *Lactobacilli* (St+Pb) alone revealed eradication of *Salmonella*, the eradication in St+Pb+Ph group was expected due to a possible synergic activity of those agents. Consequently, we suggest that the simultaneous use of a P22 bacteriophage and *Lactobacilli* probiotics mix is an interesting tool to be used as experimental treatment in chicks inoculated with *Salmonella*. Other authors^{35,36} also proposed the use of multiple biological agents as treatment for *Salmonella* by using a mix of competitive exclusion products with non-identified bacteriophages with consequent reduction in CFU number. Since the use of non-identified bacteriophages can result in the usage lentogenic bacteriophages instead of lytic ones.

Concerning weight of the chicks evaluated in this study, birds of Pb, St+Pb, Pb+Ph, and St+Pb+Ph groups revealed weight gain means higher than those of the Cr group. Such results are in accordance with those observed by other authors³⁷⁻⁴¹ who previously documented that birds receiving daily doses of *Lactobacilli* probiotics present higher weight gain rates when compared to non-supplemented birds. Additionally, those results are reinforced by Colichón *et al.*⁴² findings which demonstrated that oral administration of *Lactobacillus* in birds during the first days of life may increase weight gain up to 4.65%.

In this study, the St group revealed a statis-

tically significant weight loss in the very beginning of the experiment with subsequent weight gain. Such feature can be explained because paratyphus do not interfere in broiler chickens' productivity⁴³ since those birds usually adapt itself to the infection. Despite St+Pb and St+Pb+Ph groups were also inoculated with ST, birds in these groups did not reveal weight loss like chicks in St group. Such feature probably occurred because *Salmonella* eradication and *Lactobacilli* establishment in intestinal mucosa and lumen may have exercised beneficial effects similar to those already observed in Pb group.

Concerning morphological changes in the intestinal microenvironment, groups inoculated with *Lactobacilli* mix revealed significant increase in duodenal villi and cecal crypts size when compared to other groups. These features can be explained because probiotics stimulate proliferation of intestinal epithelial cells due to production of metabolites produced by fermentative bacterial activity.^{8,44} Despite this, other authors^{45,46} did not observe differences in size of those structures between birds supplemented with *Lactobacillus* and those without supplementation. In those cases, the results probably occurred because *Lactobacilli* strains used may not have colonized intestinal mucosa and lumen.

The St group revealed significant alterations in length of cecal crypts with gradual recovery from disease, similarly to what was observed by other authors.^{46,47} Since intestinal mucosa is a barrier that protects organisms from intestinal pathogens^{48,49} a slower proliferation of intestinal epithelial cells and/or atrophy of the intestinal mucosa structures could facilitate the invasive process by secondary pathogens after *Salmonella* infection. Villous atrophy was also observed in histologi-

cal sections, consequently justifying the diarrhea observed in birds during the first two days of experiment. Such clinical sign probably occurred due to alterations in mucosal hydrosaline equilibrium by blocking of NaCl absorption in microvilli and excretory stimulus of this salt through intestinal crypts cells.^{6,41} Despite this, body weight and intestinal microenvironment changes in were not observed St+Ph and Ph groups. Such features probably occurred because bacteriophages do not interact with eukaryotic and/or bacteria other than its specific host.²

Conclusion

Based on the findings of this study, we conclude that treatment with P22 bacteriophage and *Lactobacilli* can be effective in *Salmonella* eradication both combined and alone. We also conclude that oral administration of the P22 bacteriophage is effective in eradicating ST, promoting no perceivable alterations in birds' production or viability. Consequently, the experimental treatment here applied revealed itself as an interesting tool for ST control and eradication that can be further explored in order to be used in large scale in the Avian industry.

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